

## Genotyping of invasive Ponto-Caspian gobies in Croatia

### Genotipizacija invazivnih pontsko-kaspijskih glavoča u Hrvatskoj

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#### ABSTRACT

The biology and ecology of non-native freshwater Ponto-Caspian (P-C) gobies: monkey goby, *Neogobius fluviatilis* (Pallas, 1814), round goby, *Neogobius melanostomus* (Pallas, 1814) and bighead goby, *Ponticola kessleri* (Günther, 1861) have been studied in Croatia, but the genetic structure of populations in the Sava River catchment remains unknown. Only a single mitochondrial DNA cytochrome *b* haplotype, consistent with native Black Sea populations, has been detected within Croatian populations. Based on emerging molecular evidence, the invasive potential (e.g. upstream migration and environmental plasticity) of individual non-native gobies within the Sava River catchment, may be influenced by genetic structuring.

**Keywords:** monkey goby, round goby, bighead goby, haplotype, non-native

#### SAŽETAK

Biologija i ekologija alohtonih slatkovodnih pontsko-kaspijskih glavoča: riječnog glavočića, *Neogobius fluviatilis* (Pallas, 1814), glavočića okrugljaka, *Neogobius melanostomus* (Pallas, 1814) i keslerova glavočića, *Ponticola kessleri* (Günther, 1861) istražena je u savskom porječju republike Hrvatske, dok je genska struktura populacija ostala nepoznata. Među hrvatskim populacijama u ovom istraživanju pronađen je samo jedan mitohondrijski DNK citokrom *b* haplotip crnomorskog porijekla. Temeljem novih molekularnih dokaza, na invazivni potencijal (npr. uzvodnu migraciju i ekološku plastičnost) pojedinih alohtonih glavoča u savskom porječju, može utjecati genska struktura.

**Ključne riječi:** riječni glavočić, glavočić okrugljak, keslerov glavočić, haplotip, alohton

## INTRODUCTION

Range expansion of invasive Ponto-Caspian (P-C) gobies, since the 1990s, has extended to freshwater Eurasian and European aquatic systems as well as the North American Great Lakes (Jazdzewski and Konopacka, 2002; Jurajda et al., 2005; Poláčik et al., 2008; Brown and Stepien, 2009; Leuven et al., 2009; Neilson and Stepien, 2009b; Manné et al., 2013; Roche et al., 2013). Genes observed in native P-C populations have been transmitted (Ricciardi and MacIsaac, 2000), and haplotype diversity has increased over time due to ship ballast transfer of early life history stages (e.g. eggs, larvae and juveniles) (Brown and Stepien, 2008). Mitochondrial DNA cytochrome *b* haplotypes of monkey goby (Neilson and Stepien, 2011) and round goby (Stepien and Tumeo, 2006) have been shown to exhibit lower haplotype diversity in invading European freshwater populations than native populations of the Black Sea. However, research on microsatellites of round goby showed the same level of haplotype diversity between the native Black Sea and introduced Eurasian or North American populations (Feldheim et al., 2009). The genetic structure of invasive P-C gobies in Europe has previously been investigated using mitochondrial DNA cytochrome *b* (Stepien et al., 2005; Brown and Stepien, 2008; Neilson and Stepien 2011; Sorokin et al., 2011; Medvedev et al., 2013; Mombaerts et al., 2014; Janáč et al., 2017) and microsatellites (Vyskočilová et al., 2007; Brown and Stepien, 2008; Feldheim et al., 2009; Neilson and Stepien, 2011; Janáč et al., 2017), but molecular data specific to the Sava River catchment remains lacking.

In the Balkan countries, including Croatia, high invasive potential of P-C gobies in the Danube and Sava River basin has been identified using risk assessment protocols (Simonović et al., 2013; Piria et al., 2016a), but genetic haplotype diversity of introduced individuals remains unknown. In Croatia, three species of P-C gobies from the Danube Basin and its tributaries, the Sava and Drava Rivers: monkey goby, *Neogobius fluviatilis* (Pallas, 1814), round goby, *Neogobius melanostomus* (Pallas, 1814) and bighead goby, *Ponticola kessleri* (Günther, 1861) have been documented (Piria et al., 2011a; Piria

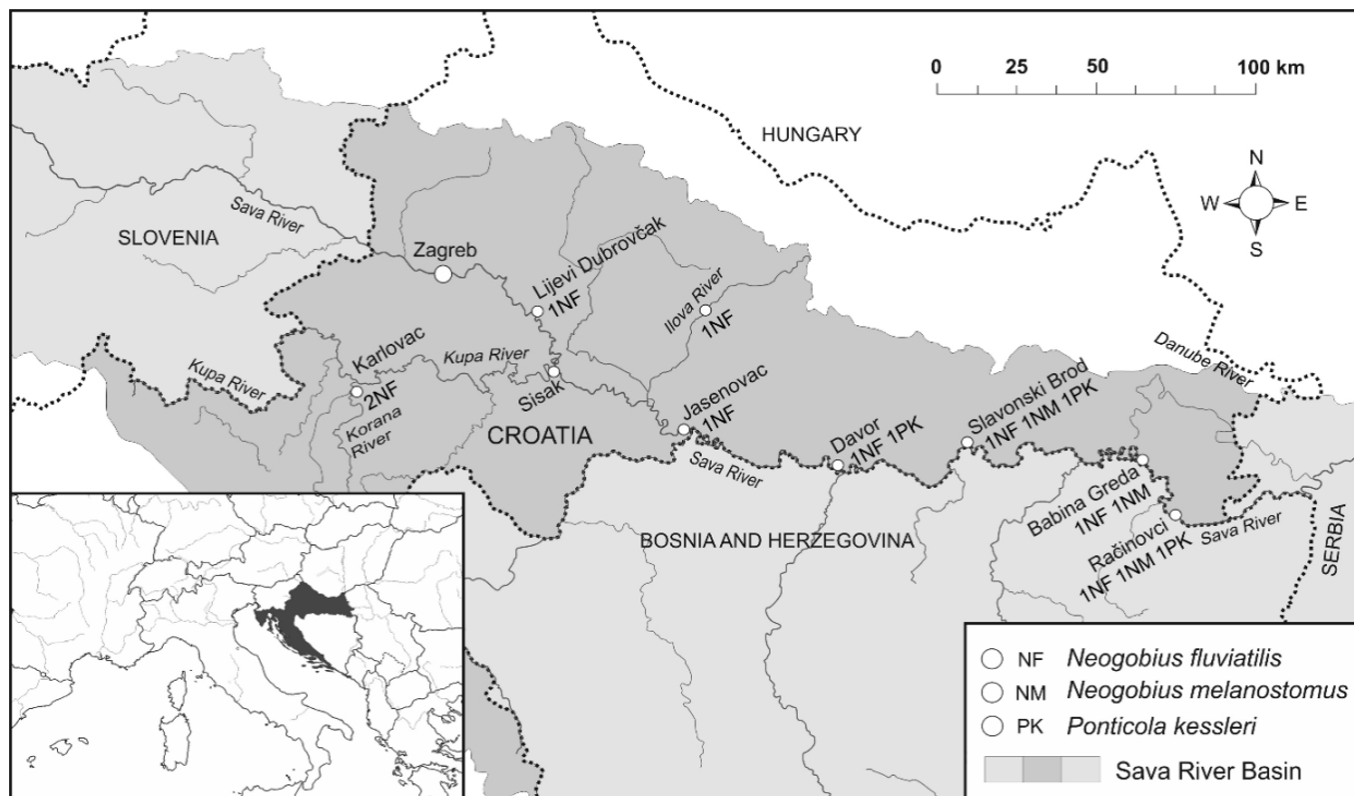
et al., 2011b; Jakovlić et al., 2015; Piria et al., 2016b) and have established self-sustaining populations (Piria et al., 2016b). Racer goby *Babka gymnotrachelus* (Kessler, 1875) is limited to the Danube River and its small tributary the Baranjska Karašica River (Piria et al., 2019). New upstream migration of P-C gobies in the Sava River has been recently reported by Simonović et al. (2017) indicating that range expansion continues. Given the lack of available data on the genetic diversity of the P-C goby populations from the Sava River, we hypothesise that haplotypes of P-C gobies from the Sava River are the same as in the non-native habitat from neighboring countries.

Thus, the aims of this study were to (1) identify haplotype diversity of the monkey goby, round goby and bighead goby from the Sava river, using sequence analysis of the cytochrome *b*; (2) compare haplotype composition to those found in its native and non-native ranges and (3) discuss their adaptation in Croatian inland waters.

## MATERIALS AND METHODS

Between 2011 and 2013, twenty-three sites were surveyed in the Sava River basin. Spatially continuous single-pass electrofishing surveys from the shore along the riverbank were undertaken during daylight hours on various types of substrates and depths ranging from ca 0.2–1.5 m. To minimize operator bias, surveys were performed using the same two-person sampling team. Electric fishing gear (Hans Grassl EL 63 II, 220/440 V, 17.8/8.9 A) with a 50 cm X 50 cm round stainless-steel anode and 10 mm mesh size netting was used. A detailed description of the sampling procedure is available in Jakovlić et al. (2015).

Upon capture, fin clips (anal fin) were taken from individual fish and preserved in Eppendorf 1.5 mL Tubes in 96% ethanol for molecular analyses. Samples were subsequently stored in a freezer at -20 °C until the time of analysis. P-C goby specimens for molecular analyses were taken from Račinovci, Babina Greda, Slavonski Brod, Davor, Jasenovac, Ilova River, Lijevi Dubrovčak and Korana locations (Figure 1).



**Figure 1.** Sampling locations of Ponto-Caspian (P-C) goby species: NF = *Neogobius fluviatilis*, NM = *Neogobius melanostomus*, PK = *Ponticola kessleri* (distribution information described by Piria et al., 2016b); Numbers indicate number of specimens used for molecular analysis at each sampling site

Genomic DNA was extracted from fin tissue of 9 monkey gobies, 3 round gobies, and 3 bighead gobies using DNeasy Blood & Tissue Kit (Qiagen Inc., USA), following manufacturer's instructions. The mitochondrial cytochrome *b* and partial threonine tRNA gene were amplified using primers AJG15 (5'-CAAAAACCATCGTTGTAATTCAACT-3') and H5 (5'-GAATTYTRGCTTTGGGAG-3') (Neilson and Stepien, 2009a). Polymerase chain reaction (PCR) was performed using a *Mastercycler personal* (Eppendorf) containing 1x PCR buffer (Fermentas), 0.2  $\mu$ M of dNTPs (Fermentas), 1.5 mM of  $MgCl_2$  (Fermentas), 1 U of *Taq*-polymerase (Fermentas), 0.2  $\mu$ M of each primer (Invitrogen), 100 ng of template DNA topped up with HPLC water to 25  $\mu$ L (Merck). Samples were initially denatured at 94 °C for 3 min followed by 40 cycles of 1 min denaturation at 94 °C, 45 s annealing at 52 °C and 1 min extension at 72 °C and followed by a final extension at 72 °C for 5 min. PCR products were purified using *QIAquick PCR Purification Kit* (Qiagen INC., USA) following the

manufacturer's instructions. Direct sequencing reactions of both strands were performed using primers: AJG15 (5'-CAAAAACCATCGTTGTAATTCAACT-3'), H15343 (5'-GGGTTATTAGATCCTGTTTCGTGTAGG-3'), L15162 (5'-GCTATGTCCTACCATGGGGCAAATATC-3') and H5 (5'-GAATTYTRGCTTTGGGAG-3') (Neilson and Stepien, 2009a). Sequences were manually adjusted by using Sequencher for Windows version 4.1.4. The obtained sequences were deposited in GenBank (Acc.no.X,Y,Z) and compared with other sequences using Basic Local Alignment Search Tool (BLAST).

## RESULTS

A total of 15 specimens were collected for molecular analysis including 9 monkey, 3 round and 3 bighead gobies (Figure 1). As a result of sequencing, for each individual monkey goby, round goby and bighead goby is detected only one cytochrome *b* and partial threonine tRNA haplotype in a total length of 1280 bp. Seven sequences of monkey goby from Račinovci, Babina Greda, Slavonski

Brod, Davor, Jasenovac, Ilova River, Lijevi Dubrovčak, as well as two sequences from Karlovac belonged to the one haplotype, haplotype NfCR1 (GenBank Accession number MG869729). Three samples of round goby from Račinovci, Babina Greda and Slavonski Brod contained haplotype NmCR1 (GenBank Accession number MG869730). Also,

three samples of bighead goby from Račinovci, Slavonski Brod and Davor contained haplotype PkCR1 (GenBank Accession number MG869731). For each of the analysed species of P-C gobies just one haplotype was determined, which means that haplotype diversity equals zero (Table 1).

**Table 1.** Cytochrome *b* diversity of the monkey goby, round goby and bighead goby populations ( $n$  = number of samples,  $n_H$  = number of haplotypes,  $h$  = haplotype diversity, N = native, I = invasive; haplotypes in this study corresponding with previously published as follow: NfCR1 = AGV7 = 5, NmCR1 = ame1, PkCR1 = Pk2 = H12); \*indicate corresponding GenBank accession number

Species	Waterbody	Location	n	nH	h	GenBank	Distribution	Haplotypes	References	
Monkey goby	Sava River			1	0	GQ444340	I	NfCR1		
	Ilova River	Croatia	1	1	0	GQ444340	I	NfCR1	This study	
	Korana River		2	1	0	GQ444340	I	NfCR1		
	Vistula River	Poland	31	1	0	GQ444336	I	1		
	Hron River	Slovakia	13	1	0	GQ444340	I	5		
	Danube River	Ukraine	-	1	0	FJ526749	N	AGV7		
	Black Sea	-	-	-	1	0	GQ444337	N	2	
		-	-	-	1	0	GQ444372	N	37	Neilson and Stepien, 2011
		-	-	-	1	0	GQ444375	N	40	
	Caspian Sea	-	-	-	1	0	GQ444434	N	99	
		Azerbaijan		6	5	0.93	-	N	-	
	Azov Sea	Ukraine	4	4	1.00	-	N	-		
	Don River	Russia	4	1	0	FJ526752	N	ANG11		
Sasyk Lake	Ukraine	1	1	0	KC886273	N	Nf1	Medvedev et al., 2013		
Volga River delta	Russia	1	1	0	GQ444411	N	76			
Round goby	Sava River	Croatia	3	1	0	EU331156	I	NmCR1	This study	
	River Waal	Netherlands	11	1	0	KJ654330	I	NSB1		
	Albert Canal	Belgium	22	2	0.17	KJ654331*	I	NSB1, 2*	Mombartes et al., 2014	
	Zeescheldt		2	1	0	KJ654331	I	NSB2		
	Baltic Sea	Poland	20	1	0	EU331156	I	ame1		
	Danube River	Serbia	45	2	0.04	EU331162*	I	ame1, 7*	Brown and Stepien, 2008	
	Dnieper River	Ukraine	15	2	0.13	EU331163*	N	ame1, 8*		

Table 1. Continue

Species	Waterbody	Location	n	nH	h	GenBank	Distribution	Haplotypes	References
Round goby	Moskva River	Russia	9	5	0.86	EU331179*	I	ame24*-28	
	Volga River		10	5	0.67	EU331185*	I	ame20, 24, 25, 29, 30*	
	Caspian Sea	Azerbaijan	5	2	0.40	EU331235*	N	ame31, 85*	
		Iran	30	11	0.77	EU331236*	N	ame31-40, 86*	Brown and Stepien, 2008
	Danube River	Slovakia	39	2	0.05	EU331178*	I	ame1, 23*	
	Black Sea	Ukraine	20	8	0.76	EU331165*	N	ame1-6, 9, 10*	
	Azov Sea		13	9	0.98	EU331174*	N	ame11-19*	
	Danube River	Germany	28	1	0	EU331156	I	ame1	Cerwenka et al., 2014
Bighead goby	Sava River	Croatia	3	1	0	KC886260	I	PkCR1	This study
	Rhine River	Switzerland	-	1	0	FJ526770	I	APT8	Kalchhauser et al., 2014
	Danube River	Serbia	-	1	0	FJ526770	I	APT8	Neilson and Stepien, 2009b
	Dniester River		11	1	0	FJ526768	N	ALC2	
	Sasyk Lake	Ukraine	1	1	0	KC886259	N	Pk1	Medvedev et al., 2013
	Danube River		1	1	0	KC886260	N	Pk2	
	Simferopol Lake		-	1	0	FJ526769	N	APT7	Neilson and Stepien, 2009b
	Black Sea basin	-	-	1	0	EU444669	N	NkeAGV3	Neilson and Stepien, 2009a
	Danube	Bulgaria	32	3	0.42	KJ605189*	N	H12, H13, H15*	
		Austria	32	2	0.28	KJ605188*	I	H12, H14*	Jánač et al., 2017
Rhine	Germany	28	2	0.29	KJ605186*	I	H12*, H14		

## DISCUSSION

For each species of goby, just one haplotype, haplotype NfCR1, NmCR1 and PkCR1 was determined, respectively. A similar observation has been reported for the Polish and Slovak monkey goby populations in the rivers Vistula and Hron (Neilson and Stepien, 2011) and German round goby populations in the Danube (Cerwenka et al., 2014), which corresponds to a relatively small number of introduced individuals (Vidal et al., 2010). Monkey goby haplotype NfCR1 = AGV7 = 5 was found in Ukraine and Slovakia (Neilson and Stepien, 2011), while the round goby haplotype NmCR1 = ame1 was found in Poland, Serbia, Slovakia, Ukraine (Brown and Stepien, 2008) as well as in Belgium, Netherlands (Mombaerts et

al., 2014) and Germany (Cerwenka et al., 2014). Bighead goby haplotype PkCR1 = Pk2 = H12 was found in Ukraine (Medvedev et al., 2013), Bulgaria, Austria and Germany (Janáč et al., 2013).

Contrary to our findings, the authors who studied Belgian round goby populations in the Rhine River basin, identified two haplotypes and determined slightly higher values of haplotype diversity, but significantly less than native Black Sea populations and the introduced American Great Lakes populations (Mombaerts et al., 2014). High haplotype diversity in the introduced American round goby populations (Dillon and Stepien, 2001) has suggested that successful invasions are a result of many multiple introductions (Bouchard et al., 2011),

thus increasing the probability of their establishment and persistence (Stepien and Tumeo, 2006; Facon et al., 2008). Furthermore, round goby invasion of the Polish and Latvian Baltic Sea is characterized by multiple introduction events and rapid adaptation to local habitats (Björklund and Almqvist, 2010). The possible reason for this adaptation is that the mitochondrial genome of the round goby (18,999 bp) is larger than most mitochondrial genomes of ray-finned fish and other vertebrates. Consequently, adaptation abilities of this species may be linked to specificity of its mitochondrial metabolism (Adrian-Kalchhauser et al., 2017). Similarly, to our results, in the Danube in Serbia (Neilson and Stepien, 2009b) and Rhine in Switzerland (Kalchhauser et al., 2016) for the bighead goby was found only one haplotype and zero value of haplotype diversity. Conversely, relatively high haplotype diversity for the bighead goby has been reported from the Danube in Austria and Rhine in Germany which suggest natural selection as well as differences in phenotypic plasticity after arrival (Janáč et al., 2017) that can lead a high tolerance to environmental variables and facilitate invasion of the species to new environments (Fox et al., 2007).

In the Sava River populations of P-C gobies appear to have found their ecological resources and demonstrate invasive potential (Piria et al., 2016b) even in this research low haplotype diversity was found. Low haplotype diversity in the introduced populations also is characteristic of other fish species, for example mosquitofish *Gambusia holbrooki* (Vidal et al., 2010), which is considered highly invasive (Simonović et al., 2013; Piria et al., 2016a). This suggests that species with certain genetic combinations favour the ability of fish to invade new habitats and successfully adapt to new ecological conditions. Indeed, relatively small number of sampled fish specimens in our research, could affect the final result, as well as Croatian war of independence in the 1990s, because at that time the Danube has reduced ship traffic, causing the closure of many of the ports (Roche et al., 2013). Furthermore, traffic on the Sava River has remained very low since the 1990s due geopolitical changes and the collapse of the economy (Roche et al., 2013). Therefore, it is possible

that only certain genetic combinations of P-C gobies succeeded without the help of ballast waters migrating upstream.

## CONCLUSIONS

Regardless of the low values of haplotype diversity and consequently small number of introduced individuals, it is likely that P-C gobies of particular haplotype are very successful in adapting to the environmental conditions of the Sava River and its tributaries. These three species are still expanding in the area and since this research represents preliminary results, detailed monitoring of their proliferation into potential new watercourses is still required.

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